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A general model of mutable connective tissues as three-phase systems has emerged from our observations on the dermis of *C. frondosa*. The model consists of a reinforcing phase (collagen fibrils); an elastomeric phase (microfibrils); and a control phase (PGs, glycoproteins, and cells). We have characterized the three phases. The native collagen fibrils are symmetrically spindle shaped, geometrically similar, and molecularly bipolar. The fibrils have proteoglycans D-periodically associated with their surfaces. The microfibrils consist mainly of fibrillin molecules covalently crosslinked into an elastic network by ε(-γ-glutamyl)lysine crosslinks. The microfibrillar networks have linear force/extension relationships up to 300% strain. The control phase contains a large glycoprotein (stiparin) that binds to and aggregates the collagen fibrils; two sulfated glycoconjugates that bind to and inhibit stiparin; and cell-secreted proteins that modulate the stiffness of the tissue. The collagen fibrils are organized into bundles by the microfibrils. Stiparin and its inhibitors determine the number of non-covalent bonds that exist between adjacent collagen fibrils. The stiffener and plasticizer, which are secreted by neurally controlled resident cells, control the stiffness of tissues by as yet undetermined mechanisms. They might work via the stiparin/inhibitor mechanism or by a parallel mechanism.

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FINAL REPORT

Grant #: N00014-91-J-1612

PRINCIPAL INVESTIGATOR: Dr. John A. Trotter

INSTITUTION: University of New Mexico

GRANT TITLE: Molecular Regulation of Mutable Collagenous Tissue

AWARD PERIOD: 1 June, 1991 - 31 July, 1997

OBJECTIVES: To determine the molecular mechanisms by which echinoderms regulate the tensile properties of their collagenous tissues.

APPROACH: The principal structural components of the sea cucumber dermis are discontinuous collagen fibrils, and its principal cellular components are secretory cells regulated by the nervous system. The cellular secretions modify the capacity of the interfibrillar matrix to transfer stress between the collagen fibrils. Modifications of this stress-transfer capacity are detected as changes in the viscous component(s) of the material using biomechanical tests. Pharmacological agents are used to probe the cellular pathways that function in tissue regulation. In addition, intact collagen fibrils are isolated from the dermis and the conditions under which they aggregate in vitro are analyzed. This fibril-aggregation assay is used to identify potential stiffening and plasticizing constituents that are extracted from the tissue.

ACCOMPLISHMENTS: We characterized the collagen molecules and fibrils from the spine ligament of the sea urchin *Eucidaris tribuloides* and the sea cucumber *Cucumaria frondosa*. The sea urchin collagen molecules are heterotrimers of composition $2\alpha1,1\alpha2$. The sea cucumber collagen molecules are homotrimers ($3\alpha1$). The sea cucumber molecules contain covalently-bound glycosaminoglycan (GAG) that plays an important role in fibril formation and structure. Both fibrils have surface-bound proteoglycans (PGs) that are periodically associated with specific locations within the D-periods. Fibrils from both species are symmetrically spindle-shaped and vary from less than 50 μm to more than 1 mm in length. Fibrils of all lengths are geometrically similar. That is, they all have the same length/maximum diameter ratio (about 2,500).

Echinoderm collagen fibrils are bipolar in a molecular sense. Half-way along the length of every fibril is a unique region, approximately 10 D-periods long, in which the polarity of the molecules is reversed. On either side of this unique region all the molecules are oriented with their amino termini toward the nearest tip. In the center of this unique region are equal numbers of molecules of each polarity. These observations, together with those related above, suggest that this central transition region with antiparallel molecular packing is a persistent site of preferred growth of the fibril.

We also characterized in the dermis of *C. frondosa* an extensive network of microfibrils. The microfibrillar network was shown to be composed primarily of the protein fibrillin. The network of fibrillin microfibrils is stabilized by covalent ε(-γ-glutamyl)lysine crosslinks produced by the action of the enzyme transglutaminase. The network has long-range elasticity (linear force/extension plots to 300% strains).

We showed that extensive extraction of the dermis of *C. frondosa* in artificial sea water (ASW) caused the tissue to disaggregate into separate collagen fibrils. We purified from the extracts a soluble glycoprotein that caused the fibrils to aggregate *in vitro*. This protein was characterized and named "stiparin." Stiparin was shown to be both necessary and sufficient to cause aggregation of fibrils in ASW.

We found that stiparin binds to fibrils but not to pepsin-digested collagen molecules. The surface PG, purified from the fibrils, inhibits stiparin's action by binding to it. A closely related PG is found in the soluble phase of the dermis. These PGs are apt to play important roles in regulating the interactions between collagen fibrils *in vivo*.

The soluble PG just described is found only in the inner dermis. A second inhibitor of stiparin was purified from the soluble phase of the outer dermis. This glycoprotein is a heterodimer of ~30 kDa subunits that are heavily sulfated and contain abundant galactose (Gal). The glycosyl moieties contain all of the sulfate and all of the inhibitory activity. This glycoprotein binds stiparin a 1:1 molar ratio.

The cells of the inner dermis of *C. frondosa* contain a protein that is released by cell-lysis and that causes the stiffening of the dermis. This protein appears after

electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) as a 38 kDa band. The purified protein causes the stiffening of dermis specimens tested *in vitro*. Hence it has been named "stiffener."

The cells of the outer dermis contain a protein that is also released by cell lysis. This protein, which has been partially purified, has an apparent MW (by SDS-PAGE) of about 10 kDa. When the partially purified protein is added to stiff dermis specimens it causes them to become markedly plastic. Hence it has been named "plasticizer."

Fresh specimens of *C. frondosa* inner dermis are plasticized in ASW that contains the calcium-chelator EGTA and stiffened when normal Ca^{2+} concentrations are restored. We showed that this effect of Ca^{2+} is not caused by a direct regulatory effect of Ca^{2+} on the extracellular matrix. Rather it is probably due to the presence of voltage-dependent Ca^{2+} channels in the membranes of dermal secretory cells, and the need for Ca^{2+} flux across these channels as part of the secretory mechanism. This concept was derived from pharmacological experiments on intact specimens of *C. frondosa*.

The dermis of a distantly related sea cucumber *A. agassizi* was found to behave similarly to that of *C. frondosa* in experiments using pharmacological agents that affect membrane potential, Ca^{2+} channels, and second messengers. It was also found to contain a stiparin-equivalent and PG equivalent in its soluble phase, and to have within its cells the equivalent of stiffener, which has been partially characterized.

CONCLUSIONS: A major objective of this project has been to characterize the molecules involved in the regulated interactions between collagen fibrils. An important component of the study has been a comparison of regulatory mechanisms in different species, because this will lead to a general model of the molecular interactions involved in regulation. The identification in *C. frondosa* dermis of stiparin, stiparin-inhibitory PG and glycoprotein, and the two proteins that plasticize and stiffen dermis, are important steps toward that objective. The subsequent identification in *A. agassizi* dermis of related but obviously different macromolecules that aggregate fibrils and inhibit fibril-aggregation provides an opportunity to investigate the general and specific macromolecular features of fibril aggregation in these distantly related species.

SIGNIFICANCE: A general model of mutable connective tissues as three-phase systems emerges from our observations. The model consists of a reinforcing phase (collagen fibrils); an elastomeric phase (microfibrils); and a control phase (PGs, glycoproteins, and cells). This model forms the basis for new investigations aimed at the development of biomimetic materials with dynamically controlled stiffness.

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